

Recombinant polypeptides for serology of malaria

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Abstract

We have evaluated 3 molecularly defined polypeptides encoded by encloned *Plasmodium falciparum* genes for their ability to serve as antigens for detecting antimalaria antibodies. The recombinant proteins correspond to (i) a conserved part of 190–200 kDa schizont merozoite surface component, (ii) the carboxy terminal part of the *P. falciparum* aldolase, and (iii) the 5:1 antigen. Antibodies were detected using enzyme-linked immunosorbent assays (ELISA) in a high percentage of sera from individuals from a malaria endemic area in The Gambia (up to 99% for some adult groups). These results were further improved, especially for detection of antimalaria antibodies in children, when a pool of all 3 polypeptides (ELISA MIXT) was used as antigen. This ELISA MIXT improves presently available assays for the detection of antimalaria antibodies directed against asexual blood stages in respect of standardization, sensitivity and specificity.

Introduction

Qualitative and quantitative determination of specific antibodies is important for measuring the impact of malaria eradication or control programmes at the population level, in malaria endemic areas. In the future antimalarial antibodies in defined areas should be monitored before, during and after vaccination trials. In developed countries, measurement of malaria antibodies can be employed to screen sera of blood donors returning from areas where malaria is endemic.

Several methods are available for the detection of antimalarial antibodies directed against asexual blood stages. Earlier tests relied on gel diffusion or on passive agglutination of sensitized erythrocytes. Other, more sensitive, tests include the indirect fluorescent antibody assay (IFA), radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) (PERRIN *et al.*, 1984). RIA and ELISA are now used increasingly for epidemiological studies because they combine sensitivity, low cost and the possibility of automation.

The major limitation of these assays is the supply of standardized reagents, particularly defined antigens. The common sources of antigens are infected red blood cells (IRBC) collected from either infected human individuals or animals (VOLLER *et al.*, 1974) and more recently asexual blood stages of *Plasmodium falciparum* produced by culture *in vitro* (SPENCER *et*

al., 1979). The IRBC extracts contain a wide array of non-parasitic antigens including erythrocyte components and break-down products of the parasite. Consequently, the use of total IRBC extract as antigen in ELISA could result in errors resulting from the high incidence of heterophile antibodies, antiglobulins or autoantibodies (ROSENBERG *et al.*, 1973) in the sera of individuals living in malaria endemic regions.

The genes encoding a number of parasite polypeptides have recently been cloned and expressed in appropriate vectors (KEMP *et al.*, 1986). Some recombinant *P. falciparum* components are now produced in high yield and purity and thus provide a source of molecularly defined polypeptides which can be used for malaria serology.

We used the following 3 *P. falciparum* recombinant polypeptides at an identical molar concentration to measure antimalarial antibodies in a human population living in an endemic area, by means of micro-ELISAs.

(i) A conserved region of the main schizont-merozoite surface protein termed 190L (GENTZ *et al.*, 1988).

(ii) Two-thirds of the 41 kDa protein (parasite aldolase) with a molecular mass of 27 kDa expressed in *Escherichia coli* (aa 112 to aa 360; U. Certa, unpublished results).

(iii) An exported antigen of the erythrocytic stage having one or more cross-reactive epitope(s) in common with the circumsporozoite protein of *P. falciparum* (HOPE *et al.*, 1985; COPPEL *et al.*, 1985), designated the 5:1 antigen.

In this investigation, 3 main points are addressed: (i) the suitability of these recombinant polypeptides for the development of serological tests, (ii) the age-dependent development of antibody to these defined polypeptides and the correlation between antibody levels against the 3 polypeptides, and (iii) the effect of the pooled polypeptides on the sensitivity of the micro-ELISA assay.

Materials and Methods

Sera

Sera samples were collected from 361 inhabitants (children and adults) of Keneba village in The Gambia during a malarial survey and stored frozen at –20°C until used. At the same time of sample collection, thick smears were also prepared for every individual and later examined microscopically for the presence of malaria parasites. All relevant information, including age, sex and previous history, was collected for every individual included in the study.

Serum samples from 5 adults in Cameroon, who had frequent bouts of malaria and an IFA antimalarial

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antibody titre of 1:6400 constituted a pool of positive sera. Similarly, a pool of 5 sera collected from individuals never exposed to malaria and negative for antimalarial antibodies by IFA served as a negative control.

Samples collected from 46 normal healthy blood donors without any past history of malaria were also included in the study as controls.

Preparation of antigens for micro-ELISA assay

Antigen 5·1. Bacterial pellets, containing the recombinant malaria antigen 5·1, were suspended in 25 mM imidazole-HCl buffer, pH 7·4, to about 4×10^{10} cells/ml. Deoxyribonuclease (DNase) I was added to 5 µg/ml. Protease inhibitors, trasyolol and phenylmethylsulphonyl fluoride (PMSF), were added to 100 units/ml and 1 mM respectively. Cells were broken in a French pressure cell at 138 000 kPa [20 000 lbf/in²]. Cell lysis, monitored by phase contrast microscopy, was greater than 98%. The crude cell lysate was centrifuged at 42 000 rpm for 60 min. More than 90% of the antigen was recovered in the supernatant as assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; TAKACS, 1979).

Solid urea was added to the supernatant to a final concentration of 6 M, and the 5·1 antigen was purified by chromatofocusing on a polybuffer exchanger column (PBE 94, Pharmacia), equilibrated with 25 mM imidazole-HCl buffer, pH 7·4, containing 6 M urea. Approximately 100 mg of protein were applied with polybuffer 74-HCl, pH 4, diluted 1:8 with water and containing 6 M urea. Antigen purification was monitored by SDS-PAGE. Fractions containing the 5·1 antigen were pooled and precipitated by dialysis against saturated ammonium sulphate at 4°C. This concentration step removed not only polybuffer (composed of amphoteric species) but also eliminated bacterial pigments which co-migrated with antigen 5·1. The precipitated antigen was pelleted by centrifugation, dissolved in phosphate-buffered saline (PBS) containing 4 M urea, and dialysed against the PBS-urea buffer to remove ammonium sulphate. The purity of the antigen preparation was assessed as 95% by SDS-PAGE.

Mice immunized with the recombinant protein produced antibodies that reacted with a 22 kDa polypeptide on immunoblots prepared from crude extracts of *P. falciparum* merozoites (data not shown).

Antigen 190 L. *E. coli* W 3110 R⁻ paste, containing the recombinant malaria protein His₆190 L, was suspended in 25 mM imidazole-HCl, pH 7·4, containing 10% glycerol and 1 mM MgSO₄. Trasyolol (100 units/ml) and DNase I (5 µg/ml) were added and the cells were broken by French pressure cell treatment at 138 000 kPa. The homogenate was centrifuged at 8000 rpm for 15 min to sediment debris and unbroken cells. Solid urea and dithiothreitol (DTT) were added to the supernatant fraction to a final concentration of 6 M and 5 mM respectively. The suspension was stirred magnetically for 30 min at 4°C and centrifuged at 42 000 rpm for 60 min to obtain the urea extract. The urea-soluble proteins were then fractionated by chromatofocusing as described above for the 5·1 antigen purification. Fractions containing

the recombinant protein were pooled and dialysed against saturated ammonium sulphate at 4°C for 16 h. For further purification the proteins precipitated by ammonium sulphate were dissolved in, and dialysed against, 0·1 M sodium phosphate buffer, pH 8·0, containing 6 M guanidine HCl. Dialysed samples were filtered through a 0·45 µm pore size membrane and applied over a Ni⁺⁺ chelate affinity column (GENTZ *et al.*, 1988), equilibrated with 0·1 M sodium phosphate buffer, pH 8·0, containing 6 M guanidine HCl. The column was washed with the same buffer with a progressive decrease of pH from 7·0 to 6·0, until absorbance at 280 nm was reduced to background level. The absorbed recombinant peptide was eluted with 0·2 M sodium acetate buffer, pH 5, containing 6 M eq. guanidine HCl. Starting with 6 g (wet weight) of *E. coli* paste, 16 mg of recombinant protein of more than 95% purity was obtained.

Antigen C 41. *E. coli* W 3110 R⁻ paste containing the recombinant malarial protein His₆ C 41 was treated in the same way as the paste containing 190 L, and C 41 was purified to >98% purity according to the procedure described above.

Assay procedure

The micro-ELISA tests were performed as described by GABRA *et al.* (1986), with slight modifications, in 96-well flat bottomed polystyrene plates (Nunc, Denmark: PS-SH-2-69620). Briefly, the plates were coated with 100 µl of antigen diluted in bicarbonate buffer (pH 9·6, 0·2 M) supplemented with 1 mg/litre of RIA-grade bovine serum albumin (BSA, Sigma Chemical Co., St Louis, Missouri, USA). The amount of each antigen was adjusted on the basis of the molecular weight of each polypeptide to a concentration of 20 nM. After adsorption of the antigens overnight at 4°C, the plates were washed twice with borate-buffered saline (BBS: 100 mM H₃BO₃, 30 mM Na₂B₄O₇·10 H₂O, and 75 mM NaCl; pH 8·4) plus 0·4% Tween-20 (BBST). Non-specific binding sites were then saturated by incubating each well with 150 µl of 0·5% bovine serum albumin (BSA) in BBS for one hour at room temperature. The plates were again washed and 100 µl of dilutions of the test sera were added to each well, followed by overnight incubation at 4°C. Each serum was tested in duplicate at dilutions of 1:100 and 1:2000 (in 2% BSA in BBST). After incubation, the plates were washed 6 times with BBST. 100 µl of a 1:300 dilution of goat anti-human immunoglobulins (λ and κ chain specific) coupled with alkaline phosphatase (Cappel Laboratories, Westchester, Pennsylvania, USA) were added to each well and the plates were left for 2 h at room temperature. After washing the plates, 100 µl of substrate solution (1 mg/ml of *p*-nitrophenylphosphate in 10% diethanolamine and 0·5 mM MgCl₂, pH 9·8) were added to each well and the plates were incubated in the dark for 2 h. In order to minimize day-to-day variation in ELISA results, the incubation time was slightly adjusted for each individual plate with respect to positive and negative controls. Positive pooled sera (at dilutions of 1/100, 1/400, 1/1600 and 1/6400) and negative pooled sera (1/100 and 1/400) were included on each plate. The ELISA values were measured at λ=405 nm using a multiscan photometer (Titerskan, Flow Laboratories, Baar, Switzerland).

Statistical analysis

The results were analysed by computer using the Statistical Package for the Social Sciences (McGraw Hill, New York, NY, USA). Correlation coefficients (*r*) were calculated using Pearson's test for pairs of variables.

Results

The age distribution of the Gambian patients in relation to detectable parasitaemia and haemoglobin levels is presented in Table 1. Most patients in the 0–10 years age group had a patent parasitaemia and relatively low haemoglobin values. With increasing age, the percentage of the population with patent parasitaemia decreased significantly and comparatively higher haemoglobin values were observed.

Micro-ELISA results for Gambian patients and blood donors using the three polypeptides as antigens are summarized in Table 2. Related to control blood donors, the Gambian patients had very high ELISA values for all 3 antigens. Slightly higher background values were observed using C 41 polypeptide compared to the other antigens. The antibody levels

varied considerably among the Gambian patients as reflected by the high standard deviation observed for the 3 antigens. At the 1:100 dilution, a high proportion of patients (24.7% for 190 L, 45.4% for 5.1, and 0.6% for C 41) had ELISA values >1.9. At a serum dilution of 1:2000, only 3.1 and 8.9 percent of patients had values >1.9 for 190 L and 5.1 respectively, while none of the patients had values >1.9 for C 41.

In order to interpret results of the ELISA, baseline levels are required. Therefore we selected as cut-off points the mean values measured in the control group of blood donors plus 2 standard deviations (SD) or plus 3 SD and determined for each age group of Gambian patients the percentage of sera with values higher than these cut-off points (Table 3). As expected, the percentage of patients with positive ELISAs for the 3 polypeptides increased with age. Fig. 1 presents the average ELISA values for the various age groups; the age-dependent increase in antibody levels for 190 L and 5.1 antigens is quite obvious, while there is only a slight increase in antibody levels to C 41.

Correlation between antibodies directed against the 3 antigens is shown in Table 4. A good correlation was observed between levels of antibodies against 190L and 5.1. In contrast, there was only a marginal correlation between antibody levels against C 41 and 5.1 and no significant correlation between C 41 and 190 L.

We next tried to optimize the assay in order to develop a sensitive assay for antimalarial antibody detection useful in epidemiological studies. The antigen source for this improved assay was a mixture of the 3 recombinant polypeptides. For the ELISAs, the antigen solutions were used at 20 nM which

Table 1. Proportion of patients from The Gambia in different age groups with patent parasitaemia and haemoglobin levels

Age group (years)	No. of patients	Percentage with patent parasitaemia	Haemoglobin level mean \pm SD* (g/dl)
0–10	128	87.4	10.76 \pm 1.91
11–20	70	60.0	12.20 \pm 1.37
21–30	49	32.0	12.92 \pm 1.86
31–40	39	30.0	12.95 \pm 2.07
>41	75	17.3	12.25 \pm 1.93

*SD=Standard deviation.

Table 2. ELISA values (absorbance at $\lambda=405$ nm) in patients from The Gambia and in controls (blood donors)

Antigens	Patients		Blood donors	
	Mean \pm SD ^a	Range	Mean \pm SD ^a	Range
190L	0.97 \pm 0.74 (0.31 \pm 0.46)	0.05–2.0 (0.02–2.0)	0.051 \pm 0.025 (0.029 \pm 0.023)	0.006–0.148 (0–0.066)
C41	0.56 \pm 0.36 (0.14 \pm 0.10)	0.07–2.0 (0–1.15)	0.145 \pm 0.043 (0.075 \pm 0.021)	0.072–0.264 (0.031–0.117)
5.1	1.38 \pm 0.73 (0.66 \pm 0.689)	0.04–2.0 (0–2.0)	0.037 \pm 0.015 (0.028 \pm 0.011)	0.014–0.097 (0.009–0.068)

^aSD=Standard deviation. First line indicates results with a serum dilution of 1:100; second line (in parentheses) indicates results with a serum dilution of 1:2000.

Table 3. Proportion of seropositive cases among the Gambian patients of various age groups reacting with three recombinant antigens

Age group (years)	No.	Seropositivity (percent) ^a					
		190L		C 41		5.1	
		Mean+2SD	Mean+3SD	Mean+2SD	Mean+3SD	Mean+2SD	Mean+3SD
0–10	128	93	81.3	78.9	70.3	87.5	85.2
11–20	70	87.1	85.7	85.7	82.9	90	90
21–30	49	95.9	93.9	91.8	91.8	98	98
31–40	39	97.4	92.3	92.3	89.7	100	100
>41	75	97.3	94.7	90.7	89.3	98.7	96

^aResults based on mean ELISA values obtained in control group plus 2 or 3 standard deviations (SD); serum dilution 1/100.

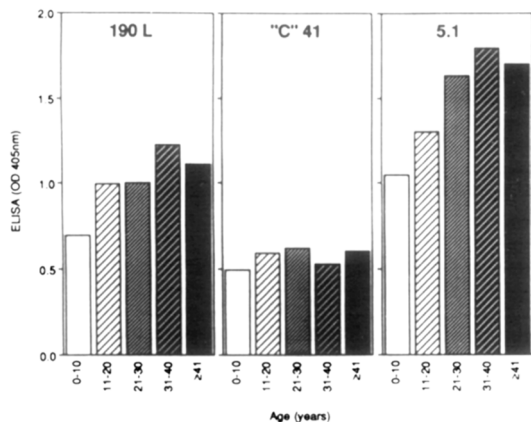


Fig. 1. Mean ELISA values for patients of various age groups using different recombinant polypeptides as antigen.

Table 4. Correlation between ELISA values obtained with different antigens

Antigens	Antigens	
	190 L	5.1
5.1	$r=0.521$ $P<0.001$	
C 41	$r=0.148$ $P<0.05$	$r=0.385$ $P<0.001$

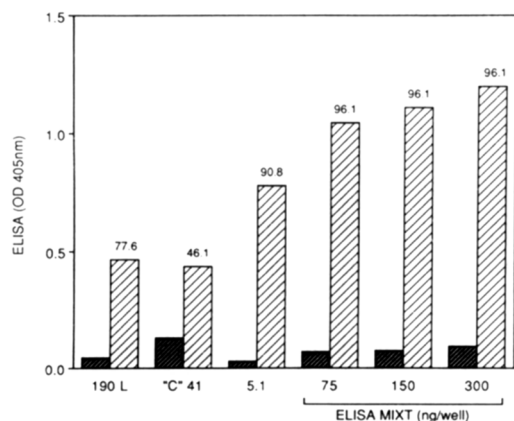


Fig. 2. Comparison of single and pooled antigens in micro-ELISA assay. ▨ Mean ELISA values for patients; ▤ control blood donors. Numbers over each column indicate percentage seropositivity at reference value of mean +3SD (serum dilution 1/100).

corresponded to 50, 55 and 45 ng/well for 190 L, C 41 and 5.1 respectively. For the new assay (ELISA MIXT) a mixture containing 40 nm of each antigen was prepared and the wells were coated with dilutions corresponding to 75 ng/well, 150 ng/well and 300 ng/well. To evaluate the sensitivity of the ELISA MIXT, 76 Gambian sera with low ELISA values in one or more of the standard assays (most of them were

children) were selected as well as 20 sera from control blood donors. The results of the various assays are shown in Fig. 2. A higher percentage of positive sera was detected by ELISA MIXT than by the standard assays. This was observed when the wells were coated with 75 ng of pooled antigens (in this mixture each single antigen was present at a lower concentration than in the assays based on a single recombinant polypeptide) and with wells coated with higher concentration of antigens. Only 3 sera were negative with the ELISA MIXT using 3 different concentrations of the pooled antigens. These 3 sera, collected from children, were also negative by IFA.

Discussion

There is a need for improved and cheap immunodiagnostic tests for the qualitative and quantitative measurement of antimalarial antibodies. The routine use of such tests requires a constant and standardized supply of malaria antigens. Conventional sources such as infected red blood cells do not meet these requirements (GABRA *et al.*, 1986; DEMEDTS *et al.*, 1987). Several genes coding for *P. falciparum* and *P. vivax* have been cloned and expressed in appropriate vectors. The sequences of the corresponding polypeptides have been determined and it has been shown that antibodies from individual patients with past malarial infection bind to some of these recombinant polypeptides (BIANCO *et al.*, 1988). No systematic serodiagnostic study has been performed with cloned antigens with the exception of that by GABRA *et al.* (1986).

We have investigated the suitability of 3 recombinant polypeptides as molecularly-defined malaria antigens for immunodiagnosis. These recombinant antigens were selected on the basis of (i) their known reactivity with antibodies from humans with past malarial infection, (ii) the fact that their amino acid sequences showed little or no variation between different isolates of *P. falciparum* (GENTZ *et al.*, 1988; CERTA *et al.*, 1988), and (iii) a high level of expression in their vectors.

All the polypeptides were suitable for the detection of antimalarial antibodies. A very low background was observed using sera of individuals never exposed to malaria and positive reactivity was detected in almost all the sera of adults of a hyperendemic area (up to 100% positive sera in some groups of adults for the 5.1 antigen). Antibodies were also reliably detected in most of the sera of children. However, there was a clear difference in the amount of antibody reaction against the three recombinant polypeptides.

Thus, levels of antibodies directed against 5.1 were the highest, followed by antibodies against 190 L and C 41. This was probably not a consequence of passive absorption of antibodies *in vivo* during an acute attack of malaria, since differences in titre were observed for both the groups with a high incidence of patent parasitaemia (children) and a low incidence (adults). This may however be the reflection of better refolding of 5.1 and 190 L and C 41, or of the presence of more epitopes able to raise an antibody response on 5.1 and 190 L.

A clear correlation was observed between antibody levels directed against 5.1 and 190 L but not between antibodies directed against these 2 polypeptides and C 41. This may have been due to very high levels of

antibody directed against 5·1 and 190 L. When a mixture of the 3 antigens was used, the percentage of positive sera increased significantly with a selection of sera poorly reactive with the individual assays. The 3 sera negative with the ELISA MIXT were also negative in the 3 assays using individual components and in IFA tests. In addition, the level of antibodies (measured by ELISA) was higher and the difference between negative and positive sera was greater.

These results suggest that an ELISA using the 3 recombinant polypeptides, available in relatively high amounts, would be useful for epidemiological studies and would also have the advantage of genuine standardization (use of molecularly defined antigens).

Additional studies are required to determine the ability of this assay to detect cross-reactive antibodies to *P. vivax* epitopes, and to compare the assay directly with IFA; however, published data concerning anti-asexual blood-stage antibody detection in children (MCGREGOR 1974; VOLLER *et al.*, 1980; GABRA *et al.*, 1986) seem to indicate that the ELISA MIXT has a higher sensitivity.

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